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DIAGNOSTIC METHOD

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DIAGNOSTIC METHOD

This invention relates to a diagnostic method and, to a nucleotide sequence encoding a Wilm's tumour (WT) WT1 antisense regulatory region, and to a method of disease prognosis based on the methylation state of the regulatory region.

Wilms' tumour (WT) is a childhood embryonal kidney tumour arising from the malignant transformation of renal stem cells. WT occurs in about 1 in 10,000 children, making it one of the commonest solid childhood tumours.

The human WT1 gene encodes a 52-54 kDa nuclear polypeptide and is genomically organised as 10 exons spanning a 60 kb chromosomal region. Intragenic deletions and mutations of the tumour suppressor gene, WT1, have been detected in approximately 10% of Wilms' tumours.

During nephrogenesis i.e. kidney development, WT1 gene expression is controlled in a highly specific manner, increasing as metanephric mesenchymal cells progress towards immature epithelial cells, and attenuating as the cells become more phenotypically mature. The inverse correlation between WT1 expression and the differentiation status of human leukaemic cells along with evidence of expression in ovary and testis and the spinal chord and brain strongly suggest that the function of the WT1 gene product may be pivotal in growth and/or differentiation in a variety of cell types. The WT1 protein, which includes

four zinc fingers, is expressed as four isoforms arising from two alternative splice sites (I and II) in the gene. Splice II occurs within the zinc finger domain, inserting or omitting

three amino-acids (KTS) between zinc fingers 3 and 4. The WT1 protein without KTS amino acids (WT1-KTS) specifically binds to the EGR site consensus sequence (5'-GCGGGGGCG-3') whereas the WT1 protein with KTS (WT1+KTS) does not. By binding to the early growth response gene (EGR) type site(s) in the promoter regions of genes such as insulin-like growth factor type II (IGF-II), platelet derived growth factor A (PDGF-A), colony stimulating factor-1 (CSF-1), and epidermal growth factor receptor (EGF-R) WT1 acts as a transcriptional repressor (reviewed in Hastie (1994) *Ann. Rev. Genet.* 28, 523-558, and Menke *et al* (1998) *Int. Rev. Cytol.* 181, 151-212).

The human WT1 promoter region has been characterised and found to belong to the family of TATA-less, CCAAT-less, GC-rich promoters with multiple responsive sites for the transcription factor Sp1. EGR/WT1 consensus sequences were also identified upstream and downstream of the major transcriptional start site (Hofmann *et al.*, (1993) *Oncogene* 8, 3123-3132) and the suggestion that these sites may allow WT1 autorepression was subsequently verified using transient transfection assays with the human promoter (Malik *et al.*, (1994) *FEBS Letters* 349, 75-78)

WT1 function is crucial in the normal development of the urogenital system, as demonstrated in WAGR (Wilms tumour, Aniridia, Genitourinary abnormalities and mental Retardation) syndrome and in Denys-Drash syndrome (DDS), diseases characterised by renal and genital abnormalities together with a predisposition to Wilms' tumour (reviewed in Coppes *et al.*, (1993) *FASEB J.* 7, 886-895).

The evidence for the involvement of WT1 in non-renal tissue differentiation is accumulating. A role in haematopoiesis is suggested by the downregulation of WT1 expression during chemically induced differentiation of HL60 cells (Sekiya *et al*, (1994) *Blood* 83, 1876-1882) and K562 cells (Phelan *et al*, (1994) *Cell Growth Differ.* 5, 677-686) Elevated WT1 expression in leukaemic cells relative to normal haematopoietic progenitor cells (Inoue *et al*, (1997) *Blood* 89, 1405-1412) and the detection of WT1 mutations in leukaemias (King-Underwood *et al*, (1996) *Blood* 87, 2171-2179; King-Underwood and Pritchard-Jones, (1998) *Blood* 91, 2961-2968) strongly implicate the involvement of the WT1 gene in leukaemogenesis. Altered WT1 expression has also been shown in breast cancers (Silberstein *et al*, (1997) *Proc. Natl. Acad. Sci. USA* 94, 8132-8137)

Furthermore, antisense WT1 mRNA transcripts with no apparent open reading frames have been detected in foetal kidney and WTs, suggesting a regulatory role for these mRNAs (Campbell *et al*, (1994) *Oncogene* 9, 583-595; Eccles *et al*, (1994) *Oncogene* 9, 2059-2063) . One such function of these mRNAs may be the formation of RNA heteroduplexes with sense WT1 mRNA, thereby modulating the finite levels of cellular WT1 protein. Previously the inventors reported the identification of an antisense WT1 promoter located in intron 1 which is activated by WT1. This effect of WT1 is reciprocal to that observed on the WT1 promoter, suggesting that the antisense promoter activity is involved in WT1 gene regulation (Malik *et al*, (1995) *Oncogene* 11, 1589-1595). In addition, it has been demonstrated that expression of ectopic exon 1 RNA can affect the cellular levels of WT1 in an *in vitro* system (Malik *et al*, (1995) *Oncogene* 11, 1589-1595;

Moorwood *et al*, (1998) *J. Pathol* **185**, 352-359), supporting a regulatory role for antisense WT1 RNAs.

The WT1 antisense transcript may upregulate the levels of WT1 protein (Moorwood *et al*, (1998) *J. Pathol* **185**, 352-359), and aberrations of the control mechanisms for antisense

RNA transcription may result in inappropriate temporal and spatial expression of WT1 protein, in turn contributing to tumourigenesis. In this regard, it is interesting to note that WT1 can increase the tumour growth rate of adenovirus-transformed baby rat kidney cells (Menke *et al*, (1996) *Oncogene* **12**, 537-546). The association between epigenetic modification of WT1 antisense regulatory regions, WT1 overexpression and renal tumourigenesis remains unclear, but preliminary studies have indicated that there is a correlation between hypermethylation of WT1 antisense regulatory regions and low WT1 protein, and the converse for hypomethylation. Interestingly, the WT1 antisense promoter locus was identified as a hypermethylated sequence in human breast cancers (Huang *et al*, (1996) *Cancer Res.* **57**, 1030-1034) and breast cancers have been shown to have decreased expression of WT1 (Silberstein *et al*, (1997) *Proc. Natl. Acad. Sci. USA* **94**, 8132-8137).

The inventors have identified a negative regulatory element (NRE) of the WT1 antisense promoter, and have demonstrated that the NRE is part of a differentially methylated region. In addition, the inventors have found a surprising correlation between the level of NRE methylation, and the disease prognosis in individual patients.

Accordingly, a first aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region comprising at least a portion of the sequence shown in

SEQ1, or at least a portion of a variant, due to base substitutions, deletions and/or additions, of the sequence shown in SEQ.1.

A second aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region comprising the sequence shown in SEQ2, or at least a portion of a variant, due to base substitutions, deletions and/or additions, of the sequence shown in SEQ.2. The WT1 antisense regulatory region may be limited to the portion of sequence shown in bold in SEQ. 2, or variants of such a sequence due to base substitutions, deletions and/or additions.

A third aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region negative regulatory element (NRE) comprising at least a portion of the sequence shown in SEQ.1 or at least a portion of a variant, due to base substitutions, deletions, and/or additions, of the sequence shown in SEQ.1. The nucleotide sequence shown in SEQ.1 may contain several WT1 antisense regulatory region negative regulatory elements.

Preferably, a nucleotide sequence according to the first, second or third aspects of the invention is a DNA or mRNA sequence.

A fourth aspect of the invention provides a method of disease prognosis in a subject diagnosed with cancer, using the differentially methylated state of specific nucleotide sequences.

The specific nucleotide sequence(s) may be one or more regulatory elements preferably one or more negative regulatory elements (NRE). The NRE sequence or sequences may be part of the WT1 gene, such that a method of disease prognosis in a subject diagnosed with cancer, comprises determining the methylation state of a negative regulatory element (NRE) of a WT1 gene in the subject, and correlating the methylation state of the NRE with the expected long-term recovery prognosis of the subject. In the case of Wilms tumours, hypermethylation of the NRE indicates that the subject has a positive long term recovery prognosis, and hypomethylation of the NRE indicates that the subject is predisposed to relapsing after treatment. However, in other cancers, hypermethylation of the specific nucleotide sequence or sequences may indicate a predisposition of the subject to relapsing after treatment, whereas hypomethylation of the specific nucleotide sequence or sequences may indicate that the subject has a positive long-term recovery prognosis.

The methylation state may be determined by restriction of the WT1 antisense regulatory region using enzymes such as *Bsh1236I*, *SpeI* and *KpnI* in combination. *Bsh1236I* is an isoschizomer of *BtsI*. *Bsh1236I* cuts at the restriction sequence CGCG only when there is no CpG methylation. Methylated sequences are not restricted by *Bsh1236I*. Therefore, the restriction pattern obtained for a nucleotide sequence which has been restricted with *Bsh1236I* gives a different band pattern depending on whether the *Bsh1236I* sites in the nucleotide sequence are methylated or not.

The methylation state may be determined using a PCR-based assay system. Such a PCR-based assay system may involve the use of sodium-metabisulphite. This has the effect of converting all unmethylated cytosine residues to uracil residues. Preferably, the

PCR reaction uses the following primers to amplify at least a portion of the WT1 antisense regulatory region:

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.

Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).

TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

The conditions used in the PCR reaction are the same as the conditions mentioned later in the specification. The PCR products obtained from the PCR reaction, as described below, may then be cloned and sequenced. The PCR products may be cloned into a vector such as pGEM-T (Promega). Alternatively, the PCR products may be sequenced directly. Once sequenced, any methylated cytosine residues will remain readable as 'C' in the nucleotide sequence, whereas unmethylated cytosines will appear as 'T' residues in the sequence.

The nested PCR reaction involves the following primers.

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.

Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).

TrN: 5'-AAACCCCTATAATTACCTCTTC-3' (nested primer).

Nucleotide sequences, and methods of disease prognosis in accordance with the invention will now be described, by way of example only, with reference to accompanying Figures

1(A) to 1(C), and SEQ.1 and SEQ.2 in which;

Figure 1(A) shows the probe used for the detection of methylation for Southern blotting; and

Figure 1(B) shows a Southern blot of three acute myelogenous leukaemia (AML) DNAs and a normal peripheral blood lymphocyte DNA; and

Figure 1(C) shows a Southern blot of DNAs from a non-tumourigenic and a highly-tumourigenic colorectal cell line; and

Figure 1 (D) shows a Southern blot of matched normal kidney and Wilm's tumour samples.

SEQ.1 shows a nucleotide sequence of the WT1 gene; and

SEQ.2 shows a nucleotide sequence of a negative regulatory element of a gene encoding WT-1.

1. Cloning and characterisation of WT1 genomic sequences

The WT1 cDNA and WT1 promoter region were cloned from a human foetal kidney cDNA library (Clontech) and a human B-cell genomic library (λ Sh2001, kindly supplied by T. H. Rabbitts, Medical Research Council, Cambridge) respectively. For each library, Plaque screen filters (Du Pont) were prepared *in situ* from 1×10^6 phage (Benton, W. D. and Davis, R. W. (1977). *Science*, **196**, 180-182). Filters were hybridized in 6x SSC (1x

SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5x Denhardt's solution, 0.5% SDS and 100 µg/ml salmon sperm DNA at 65°C. Washing was performed at high stringency (0.1x SSC, 0.5% SDS, 65°C). For the cDNA library, a partial WT1 cDNA obtained by PCR amplification was used as probe. The DNA sequence of a full-length cDNA isolated from the cDNA library was determined by the dideoxy chain terminator method (Sanger, F., *et al* (1977). *Proc. Natl. Sci. USA*, 74, 5463-5467), and a 700 bp fragment from the 5' terminus of the cDNA was used for probing the genomic library. Probes were radiolabelled with [α -³²P]dCTP (Amersham) according to the random primer method (Feinberg, A. P. and Vogelstein, B. (1983). *Biochem. Biophys. Res. Commun.*, 111, 47-54).

Genomic clones corresponding to the 5'-end of the WT1 gene were subcloned and characterised by restriction analysis according to standard methodology (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*). DNA sequences were determined by the dideoxy chain terminator method (Sanger, F., *et al* (1977). *Proc. Natl. Acad. Sci. USA*, 74, 5463-5467) and by Δ taq cycle-sequencing according to the manufacturers instructions (USB-Amersham). The functional assessment of DNA from intron 1 of the WT1 gene was carried out by transient transfection of reporter gene constructs with various WT1 intronic sequences directing gene expression (Malik, K., *et al* (1995) *Oncogene*, 11, 1589-1595).

2. Differential Methylation assays

Human genomic DNAs are purified by standard phenol-chloroform extraction procedures (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*). Based on the DNA sequence of the intronic

region (see Figure 2), digestion by restriction enzyme Bsh1236I (MBI Fermentas) has been selected to examine methylation of the intronic region. This enzyme cuts at the restriction sequence CGCG only when there is no CpG methylation; methylated sequences are not restricted. Our work has established that differential methylation is conveniently detected within a KpnI - SpeI (New England Biolabs) fragment of 850 bp, which contains 4 potential Bsh1236I sites (see Figure 1). Depending on whether these sites are methylated or unmethylated, a characteristic banding pattern is observed after digestion of genomic DNAs with a combination of KpnI, SpeI, and Bsh1236I, Southern blotting and hybridisation with a radiolabelled DNA probe (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*) defined by the KpnI and SpeI sites in the intronic sequence (Figures 1 and 2).

Figure 1(D) shows a Southern blot of matched normal kidney and Wilm's tumour samples. Samples A, B and C have no loss of heterogenicity, and a control for loss of heterogeneity is also included.

As shown in Figure 1 (D), the pattern of differential methylation successfully distinguishes between normal kidney DNA and Wilms' tumour DNA (panel A), leukaemic cells from patients with varying prognosis and normal lymphocytes (panel B) and also highly tumourigenic and non-tumourigenic colonic cell-lines (panel C). The results shown in panel C suggest that this change may be associated with the tumourigenic process and may therefore be relevant to cancers other than only Wilm's tumour.

In Wilm's tumours, hypermethylation of specific nucleotide sequences corresponds to a predicted positive long term prognosis of the subject with the Wilm's tumour, and hypomethylation corresponds to a predisposition of the subject to relapsing after treatment.

However, in other cancers, this correlation may be inverted, such that hypermethylation of specific nucleotide sequences corresponds with a predisposition to relapsing after treatment, and hypomethylation may indicate a positive long term prognosis for recovery.

3. PCR-based assay system

Tumour cells and normal cells may be distinguished by their epigenotype as previously outlined. Knowledge of the DNA sequence of the WT1 antisense regulatory region has made it possible to develop a PCR-based assay system to allow the determination of the methylation status of samples, which will require less biological material. This method involves introducing CpG dinucleotides which are not part of a restriction enzyme recognition sequence by treatment of genomic DNA samples with sodium-metabisulphite (Merck), thereby converting all unmethylated cytosine residues to uracil (Paulin, R., *et al* (1998) *Nucleic Acids Research* 8, 4777-4790). Specific regions of interest in the WT1 intronic sequence can then be amplified using primers specific for both strands of DNA. The PCR bands obtained can be directly sequenced or cloned using a commercially available vector such as pGEM-T (Promega) and analysed by DNA sequencing. Any methylated cytosine residues will remain readable as 'C' in the DNA sequence, whereas unmethylated cytosines will appear as 'T'.

Alternatively, after the first round of PCR on bisulphite-treated DNA, nested primers which include one specific for the methylated *Bsh*1236I site shown to be commonly differentially methylated (boxed in Figure 2), or one specific for the unmethylated *Bsh*1236I site (i.e. specific for C→T conversion) may be employed, permitting discrimination between methylated and non-methylated sequences by visualisation of the PCR products, i.e. if a primer specific for the methylated *Bsh*1236I site is used, a PCR product will only be observed if the *Bsh*1236I site in the sample is methylated, otherwise, no PCR amplification will occur.

Illustrative primers which may be used for methylation-specific PCR are shown below, and their hybridisation positions to the WT1 sequence are shown by arrows in Figure 2 for top-strand amplification. Allowing for C→T conversion, these are:

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.

Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).

TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

~~Typical primary amplifications are conducted with Amplitaq (Perkin Elmer) with 100 ng~~
of bisulphite-treated DNA in buffer supplemented with 3mM MgCl₂. Amplification conditions are 3 mins. denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 50°C for 30 secs, and extension at 72°C for 90 secs. A final

extension of 5 mins at 72°C completes the reaction. Secondary PCR with the nested primers employs the same conditions, but using 1/100th of the primary PCR reaction and 24 cycles.

4. Correlation of the methylation state of the (NRE) with long term disease prognosis

The inventors have detected a correlation between the methylation state of the NRE and the long term disease prognosis in subjects diagnosed and then treated for cancer. Subjects with hypermethylated NRE were treated for their cancer, responded well to treatment and made a full recovery. However, subjects who had an unmethylated NRE and relapsed were refractory to treatment.

Therefore, the methylation state of the NRE can be used as a potential early indicator of the long term diseased prognosis. Subjects who have an unmethylated NRE can be kept under closer observation for early detection of relapse. This will maximise their chances for recovery. However, the expense of such close observation post-treatment is not necessary with subjects with unmethylated NRE, as these patients are expected to respond well to treatment once any relapse has been detected by normal routine checking.

In pilot studies with AMLs, hypermethylation of specific nucleotide sequences corresponds to a predicted positive long term prognosis of the subject with the AML, and hypomethylation corresponds to a predisposition of the subject to relapsing after treatment.

However, in other cancers, this correlation may be inverted, such that hypermethylation of specific nucleotide sequences corresponds with a predisposition to relapsing after treatment, and hypomethylation may indicate a positive long term prognosis for recovery.

Therefore, decisions on the best methods of therapy to suit the subject can be made in the light of an educated expectation of how the subject is expected to respond to treatment in the event of a relapse of their cancer condition.

Therefore, it is the differential methylation that is the determinant in developing long term prognosis for subjects diagnosed with cancer.

SEQ.1

CTCGAGGATCCAGAGACGGCCTTGATCCTCTCCCCTGGGGTTTGGCCTTGGCGCTCTGAT
GGCCATTTCCACATTTTTGAGAGTTGATGCCCTTGCCCTCTCACAGCCCAAGTCTTGGGCC
AGGCCCTGCATTCTTGGGGAAGCAGCAGGAACCCTGGAAATCCAAAGAATAAACCCAGAA
TCTCGAGGGCCACCCTTGCCCACTCCAGGATAGCAGCCGGAGCGCTTCTCACATCCAAGC
TGCCCAATGAGCCTCAAGGGCTGGGTAAGATGGACCCATCTGTTTTCACTGCAAGACAAA
ACTTAAACCTGGAGATGGTGCTTCCAGGCTATATGACTTGAATCTAGGGCCCTCTCTCCA
TTGGGCTTTTTCTCCAGGGTGGAGAAGAAGGATACATTACCTACTAGTCCTGGTCCCCT
TTTAACTTTTTCTCCATGGCAGCCACGCCTGTATATTACAGAAGAATCCAGATATTTTCC
AGAAGTGTAAATACCTGCTGGCTGCAAAACCCACAGTCCCACCCCCACGACATGTGATAA
GATCCCAGGCACCAGACCTGCCCTGAAAAGGGCTGGACAAGGGACCCAAACGAAGCGACA
GAACCCAGGTTTCAAAAATCCCCTAGAAGTACTAAAAAGATAATGGCGTAGTAGTATTTT
GTGCCCCAGGGGCATGGATTGATGGTTTCTCAACCGCCTCCAAATAGCACACATGCAGA
CAGTGCTCTCGGATTCATTGTTTCTCAGTCACAGATGTTTAGATGGGTTGCCGAGTTCCA
TATTTAAAGCCCCAAGAGGGTGGTGGGTAGCGCTTCTGCATCTATGGAGTATAACTTCAA
GCCGGACCCAATCTCCAGGTTGCCCATCTCAGCTGTCCTCTTATAGACGGGGACACTGAG
ACCTAGAAACTCCCCAAAAGTAACACCAGCCTGCTAAACAAAGGTGGCGCGATCTGATCA
AAGAACACAAGCCTCAGCGATTAGTAAGTTGTCCAACGCCCCTTGAGTAGAAACACTAAT
TTACTAACTAAAAGCATAGAGTGGAGGCTTCCCTTGGGTCTGCTTGCGGTTCTCCACAG
GACAGTGATCCCAGATTCTCCCGAAGAAAAGGGCGGTTTCGATTTCTCCAAGGCTTCGCG
GGGGCCGGGTGCTCCTGGTTAAACTAAGGTAGGAGCGGCCTGAAGACGCGCGTTTAGAAG
GCGCCGGGTGAAGGCGGGCAACAAGGCAGAGCCCTTCTCCCGAGCCTTGGGCGAAGGTAC
CTCCTGCAAAAGATACACTCTGCTTCCCACGCATTCCAAAACATCCCGGTCCCTAGGCC
CTCGAGTAATTTTGCTCCAGGAAAAGCATCCGCCATTGTATTAGTAAAGCGTTTACTAAA
TTACCGAATCAAACCGAACTGGCTTAGGTTCTCAATAGCGTGGAATCCACTGAAAATAA
ATGAAGAGGGCAAACCTACAGGGGCTCCGCAGGTTCCGGTCCGCGCCGCCAGGCGAAAGA
GAGGTGGGCGGGCATCGGCGCGGGATGAGAAACCAACCTGATACTTATCGTGTGCCGAGT
TCCCTCCTTGATCCTGACTAAGCACAGCGAATAACCCTGTCCTTGTTCTAACCCAGGT
CTTGAAGAAATACTGTCCAGCTGAGCCCCGCGTTTACAAGATGAAGAGGGCGCCCCAGAT
GCGCTGAAAGAAAGGCCAAAGCTCGTGCCCTCCTTCCACTGCCTGCGGTAGAACCTGGTCC
CGCATAGCTTGAATCGGATAAGTCAAGTTCTCTTCATCCCCAGAACCTGCGTGGCCGC
CGCCTGAGCGAAGCCAGTGAAGATCCACTTCTGTATTACCATACGGGGG

SEQ.2

TTCTGCATCTATGGAGTATAACTTCAA
GCCGGACCCAATCTCCAGGTTGCCCATCTCAGCTGTCCTCTTATAGACGGGGACACTGAG
ACCTAGAAACTCCCCAAAAGTAACACCAGCCTGCTAAACAAAGGTGGCGCGATCTGATCA
AAGAACACAAGCCTCAGCGATTAGTAAGTTGTCCAACGCCCTTGAGTAGAAACACTAAT
TTACTAACTAAAAGCATAGAGTGGAGGCTTCCCTTGGGTCTGCTTGCGGTTCCCTCCACAG
GACAGTGATC

Claims

1. A nucleotide sequence encoding a WT1 antisense regulatory region comprising at least a portion of the sequence shown in SEQ.1 or at least a portion of a variant, due to base substitutions, deletions, and/or additions, of the sequence shown in SEQ.1.
2. A nucleotide sequence according to claim 1 which encodes a WT1 antisense regulatory region negative regulatory element (NRE).
3. A WT1 antisense regulatory region negative regulatory element (NRE) comprising at least a portion of the nucleotide sequence shown in SEQ.2 or at least a portion of a variant, due to base substitutions, deletions, and/or additions, of the sequence shown in SEQ.2.
4. A WT1 antisense regulatory region NRE according to claim 3 wherein the NRE comprises the sequence shown in bold in SEQ. 2, or variants of such a sequence due to base substitutions, deletions and/or additions.
5. A nucleotide sequence according to any preceding claim wherein the nucleotide sequence is a DNA sequence.
6. An RNA sequence encoded by a nucleotide sequence according to any preceding claim.
7. A method of disease prognosis in a subject diagnosed with cancer, using the differentially methylated state of a specific nucleotide sequence or sequences.
8. A method according to claim 7, comprising determining the methylation state of a negative regulatory element (NRE) of WT1 gene in a sample isolated from the subject, ~~and correlating the methylation state of the NRE with the expected long-term recovery~~ prognosis of the subject.

9. A method according to claim 8 wherein hypermethylation of the NRE indicates that the subject has a positive long term recovery prognosis, and hypomethylation of the NRE indicating that the subject is predisposed to relapsing after treatment.
10. A method according to claim 7, wherein hypomethylation of the specific nucleotide sequence or sequences indicates that the subject has a positive long term recovery prognosis, and hypermethylation of the specific nucleotide sequence or sequences indicates that the subject is predisposed to relapsing after treatment.
-
11. A method according to any one claims 7 to 10 wherein the NRE is a nucleotide sequence according to any one of claims 1 to 6.
12. A method according to any one of claims 7 to 11 wherein the methylation state is detected by restriction digest analysis.
13. A method according to claim 12 wherein at least enzyme *Bsh1236I* is used to restrict the NRE.
14. A method according to any one of claims 7 to 11 wherein the methylation state is detected using a PCR-based assay system.
15. A method according to claim 14 wherein the PCR assay system uses at least one of the the following primers to amplify a region of nucleotide sequence:
- Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.
- Tr: 5'-TAAATATCAAATTAATTCTCATCC-3'.
-
- TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).
- TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

16. A method according to claim 15 wherein the amplified nucleotide sequence is cloned and sequenced.

17. A probe comprising a nucleotide sequence according to any one of claims 1 to 6.

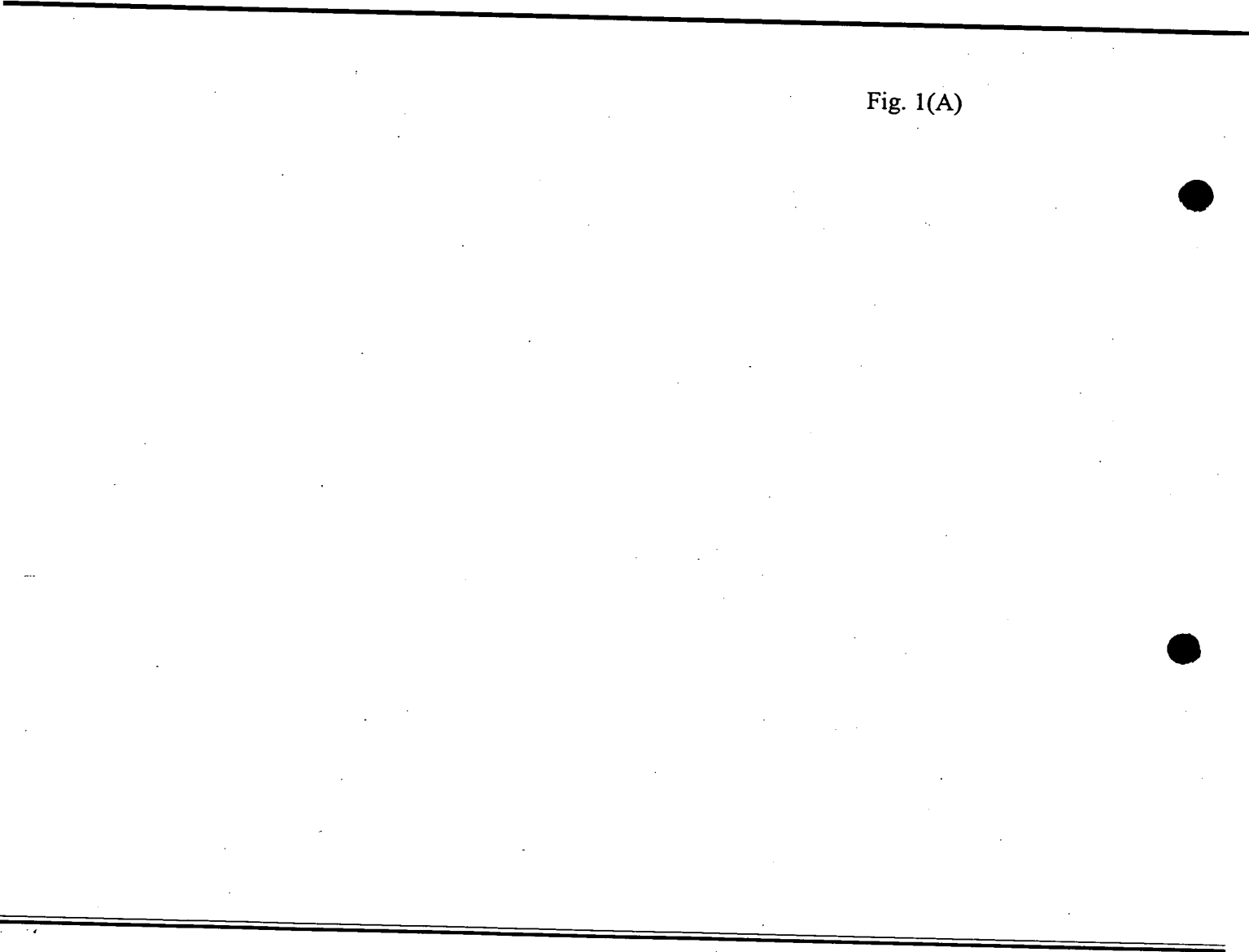
18. A diagnostic kit, assay, or monitoring method using a nucleotide sequence according to any one of claims 1 to 6 or a probe according to claim 17.

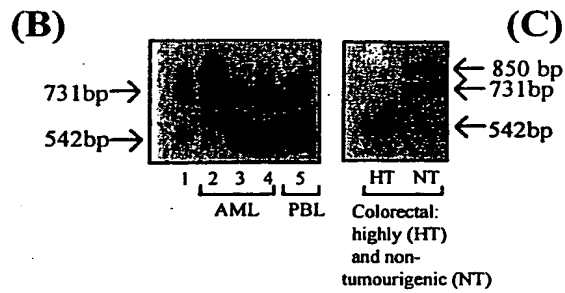
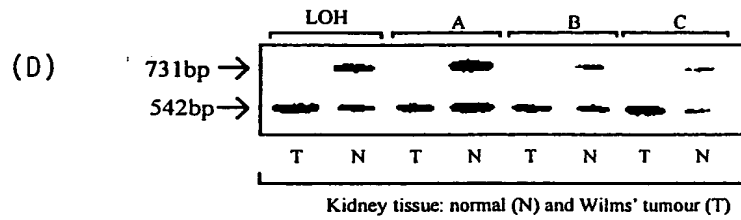
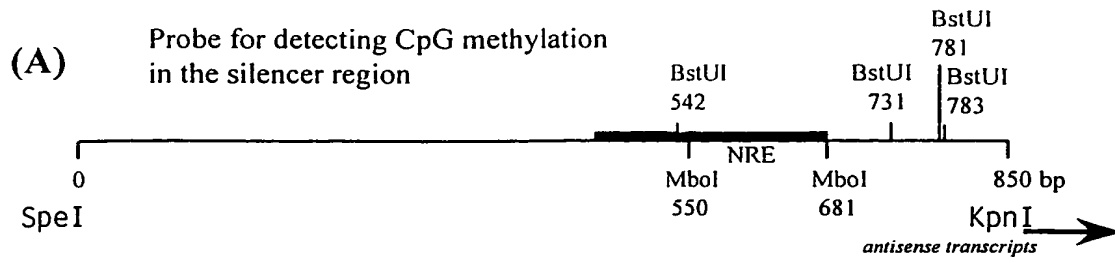
19. A diagnostic kit, assay, or monitoring method using a method according to any one of claims 7 to 16.

DIAGNOSTIC METHOD

This invention provides a method of determining the long term prognosis of a subject diagnosed with cancer, using the differential methylation state of a specific nucleotide sequence to predict the long term prognosis.

Fig. 1(A)





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Fig.2

CTCGAGGATCCAGAGACGGCCTTGATCCTCTCCCCTGGGGTTTGGCCTTGGCGCTCTGAT
 1 -----+-----+-----+-----+-----+-----+ 60
 GGCCATTTCCACATTTTGTAGAGTTGATGCCCTTGCCTCTCACAGCCCAAGTCTTGGGCC
 61 -----+-----+-----+-----+-----+-----+ 120
 AGGCCCTGCATTCTTGGGGAAGCAGCAGGAACCCTGGAAATCCAAAGAATAAACCCAGAA
 121 -----+-----+-----+-----+-----+-----+ 180
 TCTCGAGGGCCACCCTTGCCCACTCCAGGATAGCAGCCGGAGCGCTTCTCACATCCAAGC
 181 -----+-----+-----+-----+-----+-----+ 240

TGCCCCAATGAGCCTCAAGGGCTGGGTAAGATGGACCCATCTGTTTTCACTGCAAGACAAA
 241 -----+-----+-----+-----+-----+-----+ 300
 ACTTAAACCTGGAGATGGTGCTTCCAGGCTATATGACTTGAATCTAGGGCCCTCTCTCCA
 301 -----+-----+-----+-----+-----+-----+ 360
 TTGGGCTTTTTCTCCAGGGTGAGAAGAAGGATACATTACCTACTAGTCTTGGTCCCCT
 361 -----+-----+-----+-----+-----+-----+ 420
 TTTAACTTTTTCTCCATGGCAGCCACGCTGTATATTACAGAAGAATCCAGATATTTTCC
 421 -----+-----+-----+-----+-----+-----+ 480
 AGAAGTGTAATACCTGCTGGCTGCAAAACCCACAGTCCCACCCCCACGACATGTGATAA
 481 -----+-----+-----+-----+-----+-----+ 540
 GATCCCAGGCACCAGACCTGCCCTGAAAAGGGCTGGACAAGGGACCCAAACGAAGCGACA
 541 -----+-----+-----+-----+-----+-----+ 600
 GAACCCAGGTTTCAAAAATCCCCTAGAAGTACTAAAAGATAATGGCGTAGTAGTATTTT
 601 -----+-----+-----+-----+-----+-----+ 660
 GTGCCCCAGGGGCATGGATTGATGGTTTCTCAACCGCCTCCAAATAGCACACATGCAGA
 661 -----+-----+-----+-----+-----+-----+ 720
 CAGTGCTCTCGGATTCATTGTTTCTCAGTCACAGATGTTTAGATGGGTTGCCGAGTTCCA
 721 -----+-----+-----+-----+-----+-----+ 780
 TATTTAAAGCCCCAAGAGGGTGGTGGGTAGCGCTTCTGCATCTATGGAGTATAACTTCAA
 781 -----+-----+-----+-----+-----+-----+ 840
 GCCGGACCCAATCTCCAGGTTGCCATCTCAGCTGTCCTCTTATAGACGGGGACACTGAG
 841 -----+-----+-----+-----+-----+-----+ 900

ACCTAGAACTCCCCAAAAGTAACACCAGCCTGCTAAACAAAGGTGGCGGATCTGATCA
 901 -----+-----+-----+-----+-----+-----+ 960
 AAGAACACAAGCCTCAGCGATTAGTAAGTTGTCCAACGCCCCCTTGAGTAGAAACACTAAT
 961 -----+-----+-----+-----+-----+-----+ 1020
 TTACTAACTAAAAGCATAGAGTGGAGGCTTCCCTTGGGTCTGCTTGCGGTTCCCTCCACAG
 1021 -----+-----+-----+-----+-----+-----+ 1080

Bsh1236I

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1081 GACAGTGATCCCAGATTCTCCCGAAGAAAAGGGCGGTTTCGATTTCTCCAAGGCTTCGCG 1140

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Bsh1236I |

1141 GGGGCCGGGTGCTCCTGGTTAAACTAAGGTAGGAGCGGCCTGAAGACGCGCGTTTAGAAG 1200

KpnI

1201 GCGCCGGGTGAAGGCGGGCAACAAGGCAGAGCCCTTCTCCCGAGCCTTGGGCGAAGGTAC 1260

1261 CTCCTGCAAAAGATACACTCTGCTTCCCACGCATTCCAAAAACATCCCGGTCCCTAGGCC 1320

1321 CTCGAGTAATTTTGCTCCAGGAAAAGCATCCGCCATTGTATTAGTAAAGCGTTTACTAAA 1380

1381 TTACCGAATCAAACCGAACTGGCTTAGGTTCTCAATAGCGTGGAATCCACTGAAAATAA 1440

Bsh1236I

1441 ATGAAGAGGGCAAACCTACAGGGGCTCCGCAGGTTCCGGTCCGCGCCGCCAGGCGAAAGA 1500

Bsh1236I

1501 GAGGTGGGCGGGCATCGGCGCGGGATGAGAAACCAACCTGATACTTATCGTGTGCCGAGT 1560

1561 TCCCTCCTTGTATCCTGACTAAGCACAGCGAATAACCCTGTCCTTGTCTAACCCAGGT 1620

Bsh1236I

1621 CTTGAAGAAATACTGTCCCAGCTGAGCCCCGCGTTTACAAGATGAAGAGGCGCCCCAGAT 1680

1681 GCGCTGAAAGAAAGGCCAAAGCTCGTGCCTCCTTCCACTGCCTGCGGTAGAACCTGGTCC 1740

1741 CGCATAGCTTGAATCGGATAAGTCAAGTTCTCTTCCATCCCCAGAACCTGCGTGGCCGC 1800

1801 CGCCTGAGCGAAGCCCAGTGAAGATCCACTTCTGTATTACCATACGGGGG 1850

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